# The virE and virD3 Genes are Nonessential for Induction of Hairy Roots on Plants by Agrobacterium rhizogenes A4

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The *virE* gene, essential for induction of crown-gall by tumor-inducing plasmids such as pTiA6NC, was missing on the hairy-root-inducing plasmid pRiA4b, nevertheless its promoter region responsive to a plant factor remained. Introduction of the *virE* gene derived from pTiA6NC into pRiA4b-harboring *Agrobacterium* cells did not enhance the formation of hairy roots on Kalanchoe stem and leaf, indicating that the hairy-root induction does not need the *virE* function.

The pRiA4b *virD3* gene is quite different from the pTiA6NC *virD3* gene though the flanking genes *virD2* and *virD4* are conserved between the two plasmids (T. Hirayama, T. Muranaka, H. Ohkawa and A. Oka, *Mol. Gen. Genet.*, **213**, 229, 1988). A *virD3* deletion mutant of pRiA4b could induce hairy roots on Kalanchoe as well as the wild type pRiA4b, but it acted on carrot discs at an efficiency slightly lower than that of pRiA4b. The *virD3* gene was thus concluded to be dispensable for pathogenicity.

An open reading frame in which triply repeated amino-acid sequences were contained was identified between the *virD4* gene and the *virE* promoter region on pRiA4b. It was suggested to be an actual gene from Fickett analysis (J.W. Fickett, *Nucleic Acids Res.* **10**, 5303, 1982). However, it seems to be nonessential for the hairy-root induction, since its transcription was independent of a plant factor.

KEY WORDS: Tumor induction/ Virulence genes/ Ri and Ti plasmids/ Plasmid evolution/ Complementation/ Agropine-type

## INTRODUCTION

Hairy-root-inducing plasmids (pRi†) and crown-gall-inducing plasmids (pTi) are capable of conferring tumorigenic symptoms at wound sites on dicotyledonous plants upon infection by their host bacteria, *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively. This tumorigenesis is a result of the transfer of a defined DNA segment (T-DNA) from pRi/pTi into the plant nuclear genome and the constitutive synthesis of plant phytohormones directed by the T-DNA. The T-DNA also carries genes that direct the synthesis of unique amino acid derivatives called opines, by which pRi and pTi are generally classified. Imperfect 25-bp direct repeats flanking the T-DNA are requisite in *cis* for transfer. The virulence (*vir*) loci lying outside the T-DNA encode *trnas*-acting products which are involved in early events in the

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† Abbreviations used: Ap, ampicillin; bp, base pair(s); cDNA, DNA complementary to RNA; Cm, chloramphenicol; Gn, gentamicin; kb, kilobase(s) or 1000 bp; Km, kanamycin; ORF, open reading frame; pRi, hairy-root-inducing plasmid; pTi, tumor- or crown-gall-inducing plasmid; suffix r, resistance or resistant to drug; T-DNA, a DNA segment transferred from pRi/pTi to plant nuclear genomes; *vir*, virulence gene(s); vir box, the 6-bp sequence recognized by VirG protein.

plant-pathogen interaction (for review see Ref. 1). The *vir* loci of octopine-type pTiA6NC are composed of six transcriptional units which are tightly regulated (*virA, -B, -C, -D, -E,* and *-G*),[2,3] and those of agropine-type pRiA4b have a similar linear organization.[4] According to the nucleotide sequence analyses of *virA, -C, -D,* and *-G*,[4,5] however, there are some cases differing in the gene size and in the spacer length between the *vir* genes. Particularly, the *virD3* genes are quite different from each other, although the flanking genes *virD2* and *virD4* are conserved. In this report, we extended the structural analysis of pRiA4b, and found that the *virD4* gene is followed by a long open reading frame (ORF) and the *virE* promoter, but not the *virE* gene itself. In addition, we presented experimental results suggesting that *virE, virD3,* and this ORF are all nonessential for induction of hairy roots on dicots by pRiA4b.

## MATERIALS AND METHODS

### (*a*) *General procedures*

Methods for transformation with *Escherichia coli* and *A. tumefaciens,* preparation of plasmid DNA, digestion of DNA with restriction endonucleases, repair synthesis, ligation, linker-mediated ligation, gel electrophoresis with agarose and polyacrylamide, extraction of DNA fragments from gels, rapid clone analysis, radioactive labeling of DNA by nick-translation, and Southern transfer-hybridization have previously been described.[6,7] Nucleotide sequences were determined by the chain-termination procedure.[8]

### (*b*) *Culture media, antibiotics, and reagents*

L broth and L agar for *E. coli,* and YEB and YEB agar for *Agrobacterium* were described previously.[6] Murashige & Skoog medium (Flow Labo) was solidified with 1% Agar Purified (Difco). Antibiotic concentrations (mg/liter) used were: ampicillin (Meiji Seika), 20; carbenicillin (Sigma), 170 for bacterial culture and 500 for plant culture; chloramphenicol (Sankyo), 35; gentamicin (Sigma), 20; kanamycin (Meiji Seika), 20; rifampicin (Sigma), 20; and spectinomycin (Sigma), 200. Restriction endonucleases, *E. coli* DNA polymerase I and its Klenow fragment, T4 DNA ligase, RAV2 reverse transcriptase, S1-nuclease, and linker oligonucleotides were purchased from Takara Shuzo, acetosyringone from Aldrich, and [$\alpha$-$^{32}$P] dCTP from Amersham.

### (*c*) *Plasmids, bacteria, and phages*

The cosmid clones[9] used for the source of pRiA4b DNA were pBANK1329 ($Ap^rKm^r$) carrying the *Hin*dIII fragment 23b-31b region of pRiA4b, pBANK0207 ($Ap^r$) carrying the *Hin*dIII fragment 19a-14 region of pRiA4b, and pBANK0330 ($Ap^r$) carrying the *Hin*dIII fragment 13a-30a region of pRiA4b. pUAO8 ($Ap^r$) and pUAO9 ($Ap^r$) were respectively constructed by insertion into pUC18[10] of the *Hin*dIII fragments 25b and 31b which were isolated from pBANK1329. pHK210 ($Km^r$) was a clone carrying the pTiA6NC *virE* region.[11] pAO423, pAO430, and pAO736 were derived from the mini-pTi vectors pTi-1 and pTi-2.[12] pAO423 was

composed of pTi-1 with polarity I (see Ref. 12), the ColE1 replication origin, and Tn*5 kan*. pAO430 contained pTi-2 instead of pTi-1 in pAO423. pAO736 was composed of pTi-2, the pUC18 replication origin,[10] and $Gn^r$ gene. The $Gn^r$ gene was derived from the 2.6 kb *Pvu*II-*Sma*I fragment of pGmd20 (a gift of Dr. C. Sasakawa, Tokyo University). pNH156 was a recombinant between pHSG396 ($Cm^r$) (Takara Shuzo) and the 3.0-kb *Xho*I fragment[13] containing the entire *virE* operon of pTi-A6NC.

The *E. coli* strain used was JM109.[10] Pathogenic and non-pathogenic *Agrobacterium* strains were pRiA4b-harboring AR1007[4] and plasmid-free GV3101.[14] AR1029, AR1032, and AR1031[4] were Tn*3*-HoHo1[2] insertion mutants derived from AR1007. The insertion sites in the former two and the latter one were within *virD3* and *virD4*, respectively.

The single-stranded DNA phages M13mp18 and M13mp19[10] were the vectors used for sequencing.

(*d*) *Induction of and RNA preparation from* Agrobacterium *cells*

AR1007 cells were grown to 3 x $10^8$ cells/ml in YEB medium (80 ml), collected, and suspended in 40 ml of Murashige & Skoog medium supplemented with 20 mM MES-Good's buffer (pH 5.5). The cell suspension was divided into two portions (20 ml each). To one portion, acetosyringone was added to 0.2 mM (inducing conditions), and the other portion was used as a control (noninducing conditions). Cells under both conditions were cultured with aeration at 28°C for 7 hr, and harvested by centrifugation. Total RNA was prepared by the hot phenol method.[15]

(*e*) *S1-nuclease mapping and primer extension cDNA synthesis*

The procedures for S1-nuclease mapping and cDNA synthesis were previously described.[15] Six probes for S1-nuclease mapping and a primer for cDNA synthesis used were a 388-base *Cla*I-*Bal*I fragment (P1), 319–base *Apa*LI-*Nsp*(7524)V fragment (P2), 296–base *Sal*I-*Cla*I fragment (P3), 363–base *Nhe*I-*Sac*I fragment (P4), 286-base *Xho*I-*Apa*LI fragment (P5), 282-base *Hin*dIII-*Sal*I fragment (P6) and 92-base *Hin*dIII-*Pst*I fragment (P7). The former site of each fragment corresponded to the 5′-end (see Fig. 1). The autoradiograms were generated by a Fujix BA100 Bio-Image Analyzer (Fuji Photo Film).

(*f*) *Virulence tests*

Fully grown bacterial culture (about 0.1 ml containing 5 x $10^8$ cells) was injected into Kalanchoe stems with a sterile syringe, or fresh bacterial colonies were picked up by sterile toothpicks and inoculated onto young leaves of Kalanchoe. The plants were placed at room temperature, and virulence was scored as positive or negative based on the presence or absence of tumorous symptoms 3 to 5 weeks after inoculation. Another assay was carried out with carrot discs. Carrots were washed in soapy water, placed in 2% antiformin (v/v) for 15 min, rinsed with sterile water to remove excess drug, and then cut into 1-cm sections with a sterile knife. The 3 sections were placed on Murashige & Skoog agar plates. After bacteria were

spread onto the slices, the agar plates were incubated at 25°C for 4 days. The discs were then transferred onto the same fresh agar plates containing carbenicillin, and incubated in the dark at 25°C. Virulence was scored after 2 to 4 weeks as above.

## RESULTS AND DISCUSSION

### (a) *Structural analysis of the region downstream from* virD4

The pRiA4b *Hin*dIII-26 and -13a fragments on which the *virD1* to *virD4* genes are mapped have previously been sequenced[4] (see Fig. 1). Here we analyzed nucleotide sequences of the downstream region, the *Hin*dIII-25b fragment and a part of the *Hin*dIII-31b fragment. It was confirmed that the *Hin*dIII fragments 13a, 25b, and 31b are contiguous in this order, by the sequencing of appropriate restriction fragments across their junctions. The sequences thus determined are presented in Fig. 2. Nucleotides were numbered from the left end residue of the *Hin*dIII-26 fragment, consistently with the numbering in the previous report.[4] An ORF (nucleotide 7866 to 10388) was found downstream from *virD4*. This ORF could code for an amino-acid sequence of about 120 residues which was intramolecularly repeated three times (indicated by arrows in Fig. 3, left). The probability of coding sequences for this ORF were estimated by Fickett's analysis,[16] which is based on the fact that nucleotides tend to be repeated with a periodicity of three in protein coding sequences. As a result, high probability values for both the entire ORF region (0.97) and the repeated region of nucleotide 7866 to 9116 (1.03) were obtained, suggesting that this ORF actually codes for a protein. The presence of a potential ribosome binding site[17] at nucleotide 7852 to 7859 further suggests translation of this ORF. This ORF was thus temporary termed *virD5*. The corresponding DNA

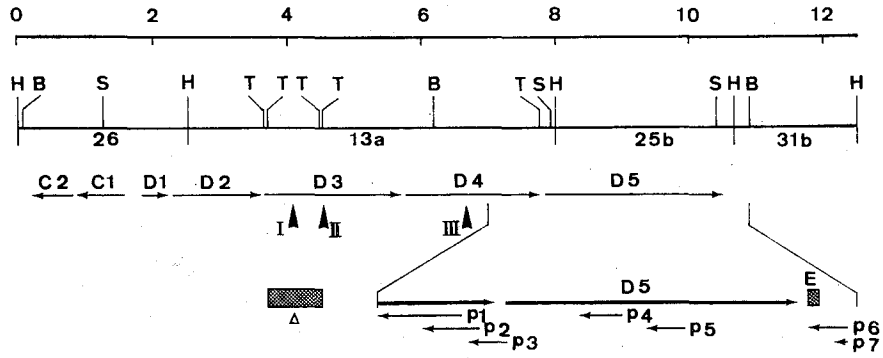


Fig. 1. Structure of the pRiA4b *Hin*dIII fragment 26–31b region. The scale in kb at the top is the distance from the left end of *Hin*dIII–26 fragment. Relevant cleavage sites are shown by vertical lines (B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; and T, *Tth*111I). The *vir*-coding regions are indicated by horizontal arrows in the 5′ to 3′ direction (D1 to D5, *virD1* to *virD5*, respectively; C1, *virC1*; and C2, *virC2* (Ref. 4 and this paper). Up arrows I, II, and III represent the Tn*3*-HoHol insertion sites in AR1032, AR1029, and AR1031, respectively. In the enlarged portion, the locations of probes used for S1-nuclease mapping (P1 to P6) and of primer (P7) used for cDNA synthesis are indicated by arrows in the 5′ to 3′ direction. Shaded bars show the locations of the *virD3* deletion in pNH131 ("Δ") and of the *virE*-promoter sequence similar to that of pTiA6NC ("E").

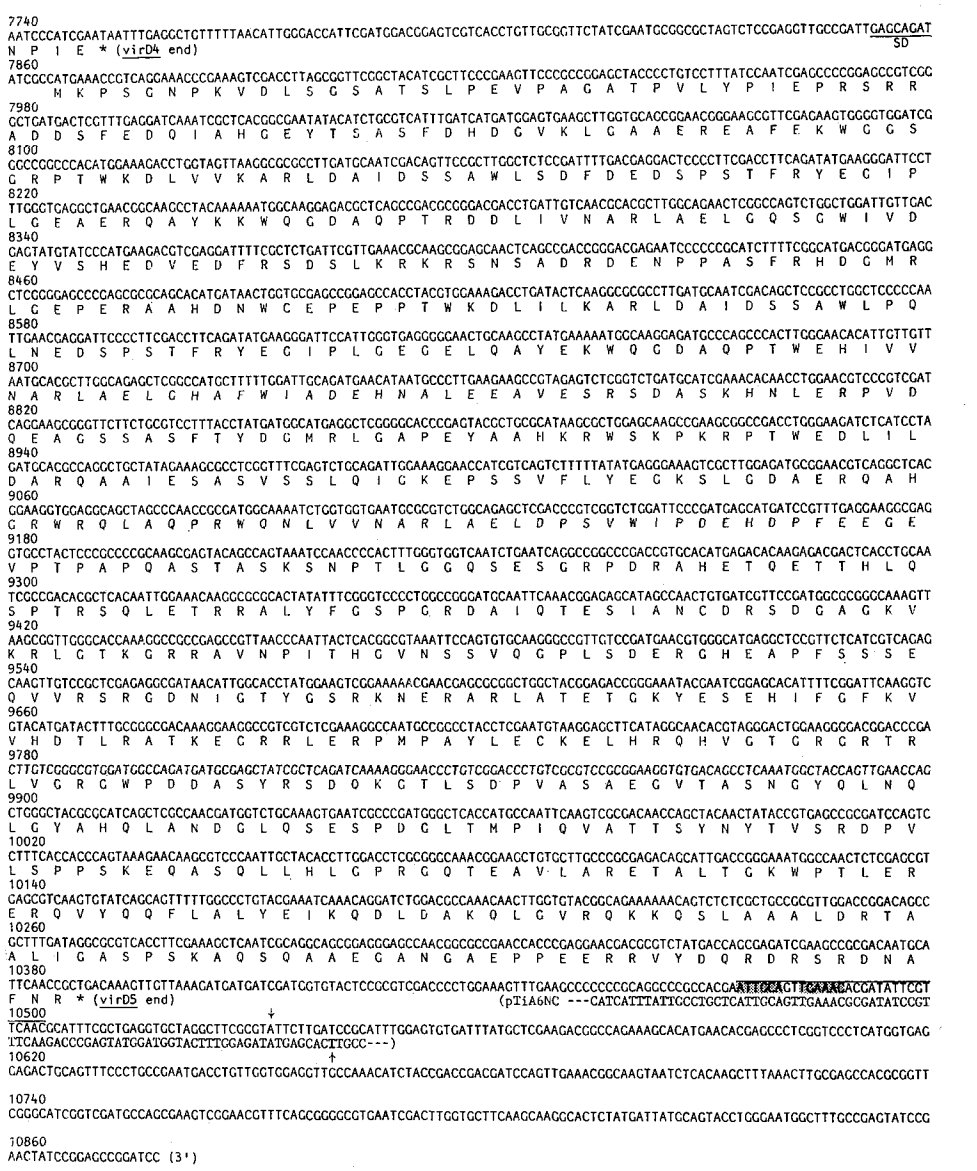


Fig. 2. Nucleotide sequence of the DNA region downstream from pRiA4b *virD4*. Nucleotides are numbered from the left end residue of *Hin*dIII-26 fragment, and the numbers are shown at the left side, corresponding to the scale in Fig. 1. They are consistent with the numbering in the previous report.[4] The sequence of nucleotide 7740 to 8057 is from Ref. 4. The predicted amino-acide sequences of VirD5 and the C-terminal portion of VirD4 are shown under the nucleotide sequence. An asterisk indicates a termination codon. The potential ribosome binding sequence (SD) and the *virE* promoter region similar to that of pTiA6-NC are marked by an underline and an upperline, respectively. For comparison, the nucleotide sequence of the pTiA6NC *virE* promoter region is shown in parentheses at the corresponding position. The inverted repeats of the vir box (ATTGCANNTGAAAC) are shaded, and the starting site of inducible *virE*-RNA is indicated by a down arrow for pRiA4b and by an up arrow for pTi-A6NC.

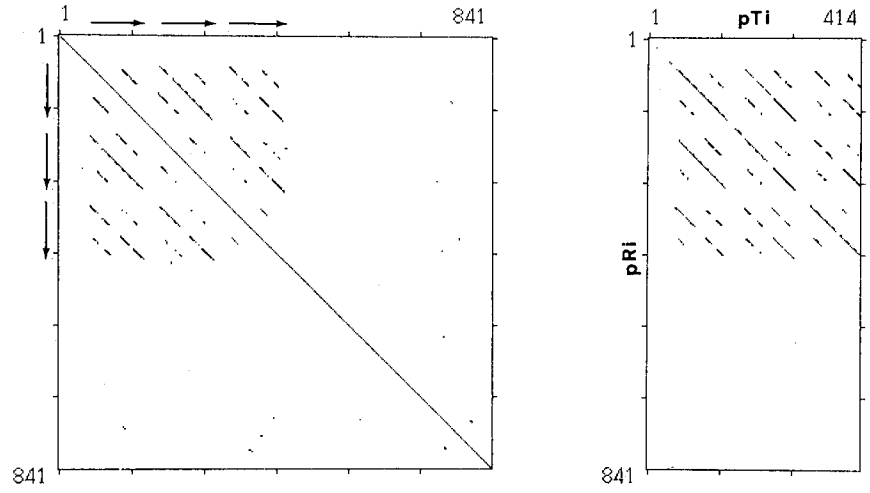


Fig. 3. Dot matrix analysis for intramolecular amino-acid sequence similarity of VirD5 protein (left) and for intermolecular amino-acid sequence similarity between the pRiA4b VirD5 protein and the putative pTiA6NC protein translated from ORF6 (right). A dot represents the presence of 6 identical residues in each 12 consecutive residues.

region on pTiA6NC has been partly sequenced[18] and the predicted amino-acid sequence from it (ORF6 in Ref. 18) displayed high similarity to that of the N-terminal portion of pRiA4b VirD5 protein (Fig. 3, right).

About 30 bp of sequence homologous to the pTiA6NC *virE* promoter region[13] (marked by an upperline in Fig. 2) were identified, but no extensive similarity to the pTiA6NC *virE* region was found. The 30-bp sequence contained the 6-bp inverted repeats for recognition by VirG protein ($TG^{A}_{T}AA^{C}_{T}$; vir box), essential for transcription induced by a plant factor.[15] No sequence for the pRiA4b *virE* gene was present in the 300-bp region downstream from the vir box, though the pTiA6NC *virE* gene appears in the corresponding region.[13,19] To rule out the possibility that *virE* was translocated elsewhere during the evolution of pRiA4b, Southern hybridization experiments were done. When the 2.5-kb *Eco*RI-23 fragment carrying the 5′ half of pTiA6NC *virE* and its upstream region purified from pHK210 was used as a probe, it hybridized to the downstream region of pRiA4b *virD4*, the *Hin*dIII-25b fragment (Fig. 4a) (see Ref. 4). On the other hand, when a subfragment of the above probe, the 1.2-kb *Sph*I-*Eco*RI fragment, carrying only the internal coding region of pTiA6NC *virE* was a probe, a hybridization signal was detected with neither total DNA prepared from AR1007 nor pBANK0330 DNA (Fig. 4b). These results suggest the lack of *virE* on pRiA4b, and are consistent with a recent report[20] that the pTi *virE* probe does not hybridize to the downstream region of *virD4* on pRiHRI very close to pRiA4b.

On the basis of sequence similarity among the *vir* genes of pRiA4b, pTiA6NC, and nopaline-type pTiC58, we have previously offered the hypothesis that a set of *vir* genes has evolved from a common ancestral set of *vir* genes and that the phylogenetic distance between pRiA4b and pTiC58 is the closest among the three relations.[4,5]

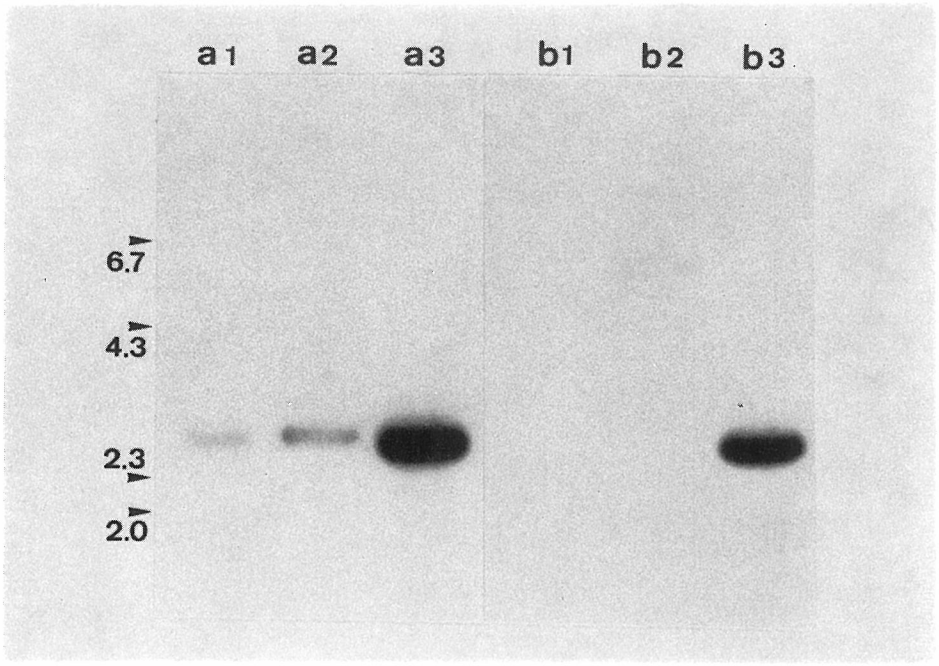


Fig. 4. Southern blot hybridization. DNA was digested with restriction endonucleases, electrophoresed on 0.7% agarose gel, Southern-transferred to a nitrocellulose membrane, and then hybridized with the $^{32}$P-labeled probe of 2.5 kb *Eco*RI-23 fragment isolated from pHK210 (a) or 1.2 kb *Sph*I-*Eco*RI subfragment (b). (1) *Hin*dIII digest of DNA isolated from AR1007; (2) *Hin*dIII digest of pBANK0330 DNA; and (3) *Eco*RI digest of pHK210 DNA. The band positions of *Hin*dIII fragments derived from lambda phage DNA are shown at the left side for size markers in kb. A faint band in (b2) corresponds to that of the vector of pBANK0330, which appeared due to the trace contamination of vector DNA in the probe preparation.

Nevertheless pTiC58 does carry the *virE* gene close to that of pTiA6NC,[20,21] indicating that pRiA4b *virE*, if it existed, could be detected by hybridization. Therefore, pRiA4b *virE* was concluded to be lost during evolution.

(*b*) *Transcription from the* virD5-virE *region*

Transcription on the region downstream from *virD4* under inducing and noninducing conditions was analyzed by S1-nuclease mapping. RNA was isolated from AR1007 cells grown under inducing and noninducing conditions (see Materials and Methods). The $^{32}$P-labeled probes used are shown in the enlarged portion of Fig. 1 (P1 to P6). The probes that had been protected by RNA from S1-nuclease digestion were electrophoresed, and the RNA starting positions and relative amounts of RNA were estimated from the band position and intensity, respectively. When the P1 to P3 probes corresponding to the 5′ and upstream portion of *virD5* were used, bands were detected only under the inducing conditions (Fig. 5a-c). Since the band positions corresponded to the respective probe sizes, the transcription was initiated upstream from the P1 probe region, presumably in the *virD1* promoter region.[15] In contrast, when the P4 to P6 probes corresponding to the downstream

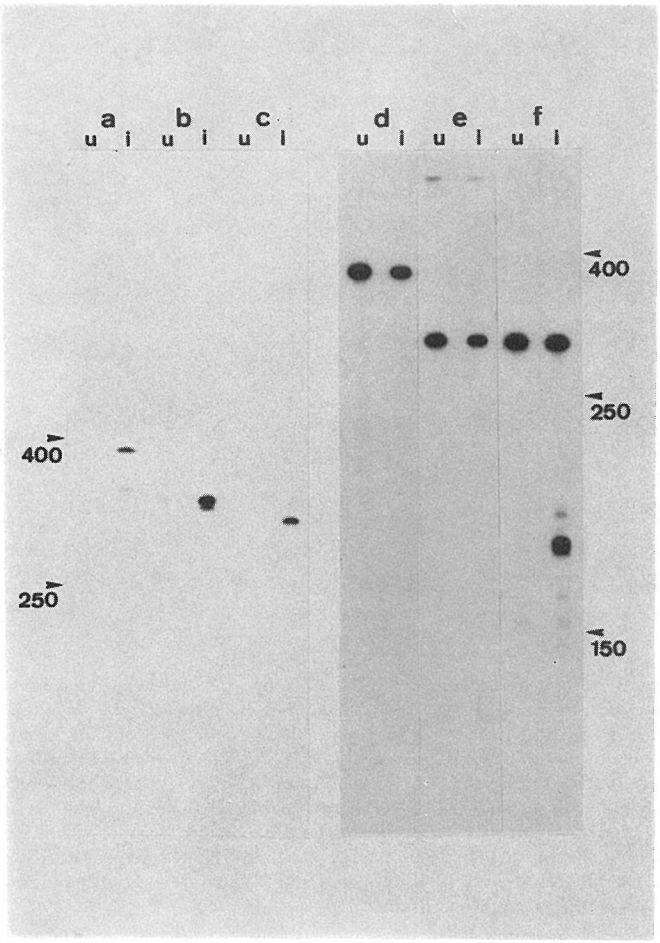


Fig. 5. Electropherogram for S1-nuclease mapping. RNA prepared from induced and uninduced cells (shown by "i" and "u", respectively) were annealed with each of six $^{32}$P-labeled probes (P1 to P6), treated with S1-nuclease, and then electrophoresed on an 8% polyacrylamide-urea gel. The size markers (in bases) from a sequence ladder are indicated at both sides. The probes used in (a) to (f) were P1 to P6, respectively. Electrophoreses of (a)-(c) and (d)-(f) were done separately.

regions were used, bands of nearly constant intensity under the inducing and noninducing conditions appeared at the positions corresponding to the respective probe sizes (Fig. 5d-f). These results suggest that the inducible transcription initiated at the promoter for *virD1* can read through into the *virD5* region but stops before the P4 probe region, and that the constitutive transcription is initiated at a site between the P3 and P4 probe regions, possibly at an unidentified promoter for *virD5*. Therefore, *virD5* is likely not to be a member of the *virD* operon. Since all of the pRi/pTi genes required for virulence that have been so far examined are inducible by plant factors,[2,15,22] *virD5* is likely to be nonessential for pathogenicity.

Another transcription that was inducible was identified with the P6 probe (Fig. 5f). To pinpoint this RNA starting site, more precise S1-nuclease mapping with the

P6 probe and primer extension with the P7 primer (see Fig. 1) were done with juxtaposition of a sequence ladder. Electrophoresis with cDNA elongated from the primer gave a single band, but one with the probe protected by RNA from S1-nuclease produced several consecutive bands involving the above band (Fig. 6). This difference might be due to imprecise cutting by S1-nuclease at the boundary between double- and single-stranded DNA regions[23] or overdigestion by S1-nuclease. Therefore, we considered that the 3′-end of the cDNA corresponded to the 5′-end of transcript, and assigned the RNA starting site on the DNA sequence as indicated by a down arrow in Fig. 2. This site approximately corresponded to the mRNA starting site for pTiA6NC *virE*[19] shown by an up arrow in Fig. 2. Therefore, it was concluded

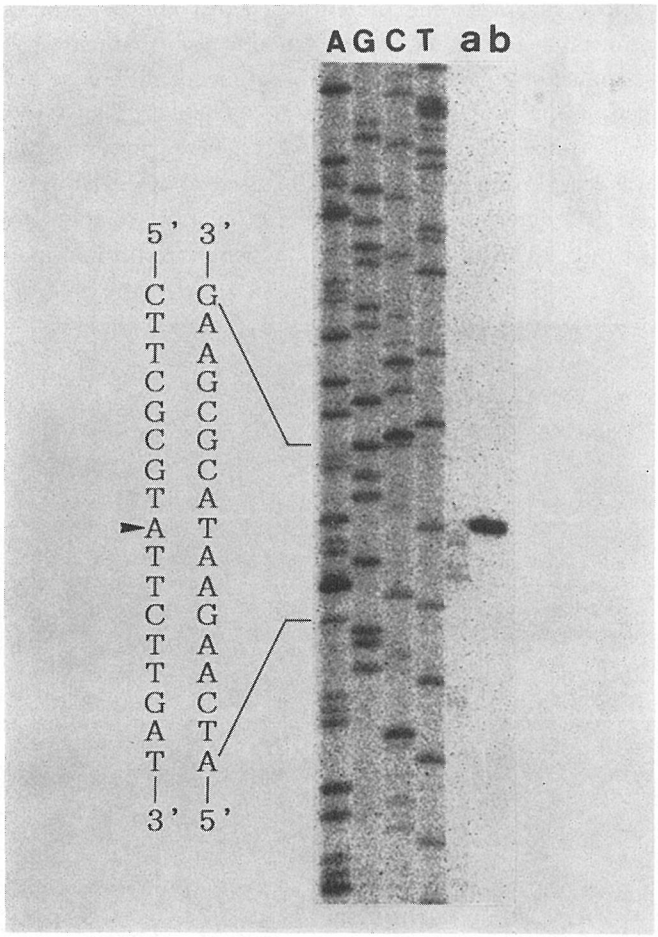


Fig. 6. Electropherogram for S1-nuclease mapping (a) and primer-extension cDNA (b) with juxtaposition of a sequence ladder (A, G, C, T). The RNA used was prepared from induced cells. The DNA sample for the sequence ladder was made by the chain termination method using single-stranded M13mp18 DNA carrying the corresponding DNA region and the P7 primer. The sequences of both strands in the relevant regions are shown on the left side. The 5′-end of transcript deduced from the primer-extension experiment is indicated by an arrow.

that the promoter for pRiA4b *virE* is inducibly active, but the *virE* gene itself was missing and the -10 region of promoter was not conserved.

(*c*) *Effects of the presence of* virE *on hairy-root induction*

The *virE* mutants of pTiA6NC become avirulent on various dicotyledonous plants.[2] However, pRiA4b missing *virE* can induce hairy roots on a wide variety of dicots. The grant of *virE* to pRiA4b-harboring AR1007 may therefore enhance hairy-root induction. To examine this, the 3.0-kb *Xho*I fragment carrying pTiA6NC *virE* through pNH156 was inserted in the mini-pTi vectors pAO736 and pAO423 in both orientations, and the resulting recombinant plasmids (pAO576 and pAO578 from pAO736; pAO579, pAO580 and pAO581 from pAO423; pAO581 was the same as pAO580 except for the presence of extra one copy of the 3.0-kb *Xho*I fragment) were introduced into AR1007. These transformant strains were tested for virulence with Kalanchoe stem and leaf. No significant difference between the presence and absence of *virE* was noticed (Fig. 7). Therefore, hairy-root induction by pRiA4b seems not to need the function of *virE*. This is presumably the reason why pRiA4b *virE* was lost during evolution as discussed above. However, a possibility that pRiA4b carries a determinant of function equivalent to that of *virE* cannot be completely ruled out. The ability of pRi1855 (whose restirction map is identical to

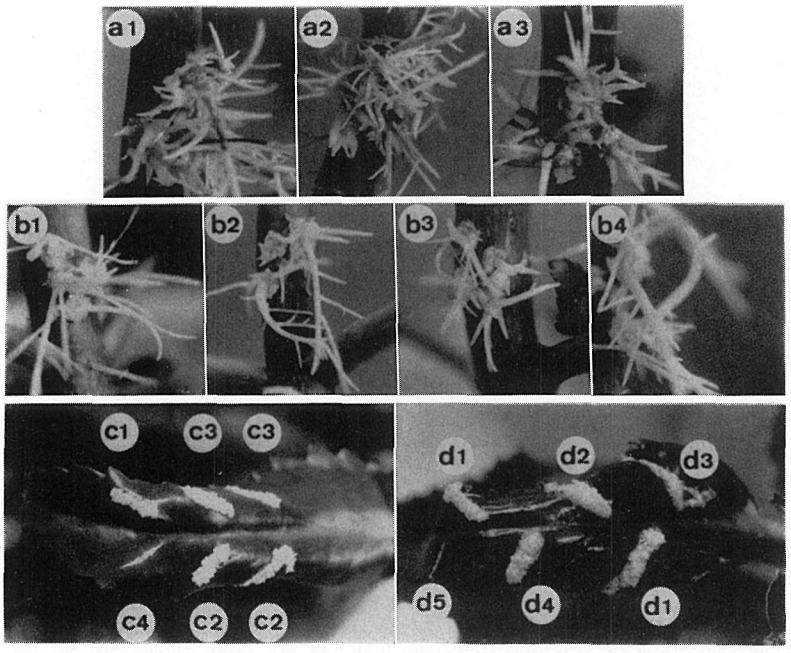


Fig. 7. Virulence test with Kalanchoe stem and leaf of various *Agrobacterium* strains: AR1007, (a1); AR1007 (pAO576), (a2); AR1007(pAO578), (a3); AR1007, (b1); AR1007(pAO579), (b2); AR1007(pAO580), (b3); AR1007(pAO581), (b4); AR1007, (c1); AR1007(pAO576), (c2); AR1007(pAO578), (c3); GV3101 (c4); AR1007, (d1); AR1007 (pAO579), (d2); AR1007(pAO580) (d3); AR1007 (pAO581), (d4); and GV3101, (d5). The tests of (a) to (d) groups were done separately.

that of pRiA4b) to restore virulence on tomato of octopine-type pTiAch5 *virE* mutants as well as *virB* and -*C* mutants may relate to this possibility.[24]

(*d*) *Phenotype of* virD3 *mutants*

Although most *vir*-coding regions are highly conserved between pRiA4b and pTiA6NC, the entire *virD3* and the latter half of *virD2* are quite different from each other.[4] The extensive divergence in *virD2* was attributed to the absence of an enzymatically active site in the C-terminal half of VirD2 protein.[4,25] As to the *virD3* gene, we previously isolated several avirulent mutants with Tn*3*-HoHol insertion within *virD3*. However, since Tn-insertion mutants generally show polar effects, and since the downstream *virD4* is essential for pathogenicity,[2,4] the requirement of *virD3* for virulence cannot be assessed from this type of experiment alone. Therefore, the *Sal*I-*Bam*HI fragment (nucleotide 1182 to 10879) carrying the *virD*1 to *virD5* region was cloned in both orientations into a mini-pTi vector pAO430 (pNH112 and pNH113). A deletion within *virD3* was introduced on pNH112 by ejection of the *Tth*111I fragment of nucleotide 3624–4517 (pNH131). Each of these three recombinant plasmids was then introduced into *Agrobacterium* strains (AR1029, AR1031, and AR1032) carrying a Tn insertion within *virD3* or *virD4* (see Fig. 1), and their pathogenicity was examined with Kalanchoe stems and carrot discs. The results are summarized in Table 1, and representative photographs are shown in Fig. 8. Complementation by pNH131 to the *virD3* and *virD4* insertion mutations occurred almost equally with that by pNH112 and pNH113, though on carrot discs, the ability of pNH131 to complement was slightly lower than that of pNH112 and

Table 1

| *Agrobacterium* strain | Genotype (resident pRiA4b)/(introduced mini-pTi) | Virulence on Kalanchoe | Virulence on Carrot |
|---|---|---|---|
| AR1007 | *vir*$^+$ | ++ | ++ |
| GV3101 | *vir*$^-$ | — | — |
| AR1029 | *virD3*::Tn | — | — |
| AR1029 (pNH112) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | ++ |
| AR1029 (pNH113) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | ++ |
| AR1029 (pNH131) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^-$*4*$^+$*5*$^+$ | ++ | + |
| AR1032 | *virD3*::Tn | — | — |
| AR1032 (pNH112) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | + |
| AR1032 (pNH113) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | + |
| AR1032 (pNH131) | *virD3*::Tn/*virD1*$^+$*2*$^+$*3*$^-$*4*$^+$*5*$^+$ | ++ | + |
| AR1031 | *virD4*::Tn | — | — |
| AR1031 (pNH112) | *virD4*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | ++ |
| AR1031 (pNH113) | *virD4*::Tn/*virD1*$^+$*2*$^+$*3*$^+$*4*$^+$*5*$^+$ | ++ | ++ |
| AR1031 (pNH131) | *virD4*::Tn/*virD1*$^+$*2*$^+$*3*$^-$*4*$^+$*5*$^+$ | ++ | + |

Virulence test with Kalanchoe stems and carrot discs. "+, ++, +++" and "—" represent the presence and absence of tumorous symptoms, respectively. "+", "++" and "+++" indicate approximate relaive intensity of tumorigenesis.

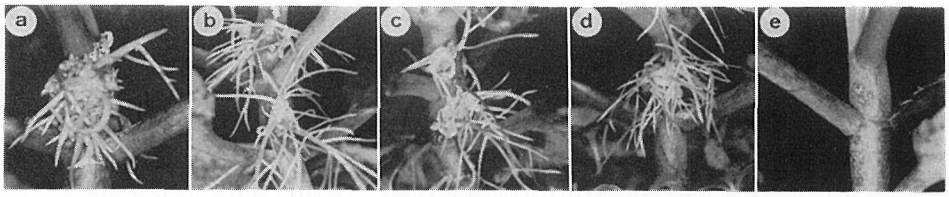


Fig. 8. Virulence tests on Kalanchoe stems of AR1007 (a), AR1029(pNH131) (b), AR-1032(pNH131) (c), AR1031(pNH131) (d), and AR1029 (e).

pNH113. Thus it was concluded that the *virD3* is nonessential for hairy-root induction, at least on Kalanchoe stems and carrot discs.

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